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DHX15 is up-regulated in castration-resistant prostate cancer and required for androgen receptor sensitivity to low DHT concentrations

Yadong Xu, BS[1,2,3], **Qiong Song, BS**[3,4], **Laura E. Pascal, PhD**[3], **Mingming Zhong, PhD**[3], **Yibin Zhou, MD**[3,5], **Jianhua Zhou, BS**[3], **Fang-Ming Deng, MD, PhD**[6], **Jiaoti Huang, MD, PhD**[7], **Zhou Wang, PhD**[3,8,9]

[1]Department of Urology, The Second Xiangya Hospital of Central South University, Changsha, China

[2]The Third Xiangya Hospital of Central South University, Changsha, China

[3]Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

[4]Center for Translational Medicine, Guangxi Medical University, Nanning, China

[5]Department of Urology, The Second Affiliated Hospital of Soochow University, Suzhou, China

[6]Department of Pathology, NYU School of Medicine, New York, New York

[7]Department of Pathology, Duke University School of Medicine, Durham, North Carolina

[8]UPMC Hillman Cancer Center, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

[9]Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Abstract

Background: DHX15 is a member of the DEAH-box (DHX) RNA helicase family. Our previous study identified it as an AR coactivator which contributes to prostate cancer progression.

Methods: We investigated DHX15 expression in castration resistant prostate cancer specimens and the influence of DHX15 on the responsiveness of prostate cancer cells to DHT stimulation. We also explored the role DHX15 played in enzalutamide resistance and the interacting domain in DHX15 with AR. DHX15 expression level in human CRPC specimens and prostate cancer specimens was detected by tissue microarray (TMA) immunostaining analysis. Colony formation assay was performed to determine the proliferation of cells treated with enzalutamide or DHT.

Correspondence Zhou Wang, Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, PA. wangz2@upmc.edu.

CONFLICT OF INTEREST

The authors declare no competing interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

siRNAs were used to knockdown DHX15. The interactions between DHX15 and AR were detected using co-immunoprecipitation assay.

Results: The expression level of DHX15 was upregulated in human CRPC specimens compared with hormone naïve prostate cancer specimens. DHX15 knockdown reduced AR sensitivity to low DHT concentrations in C4–2 cells. Inactivation of DHX15 sensitizes the enzalutamide treatment in C4–2 cells. Deletion mutagenesis indicated that DHX1 5 interacts with AR through its N terminal domain.

Conclusions: These findings suggest that DHX15 contributes to prostate cancer progression. DHX15 is required for androgen receptor sensitivity to low DHT concentrations and contributes to enzalutamide resistance in C4–2 cells. Targeting DHX15 may improve the ADT treatment.

Keywords

androgen receptor; castration resistant prostate cancer; DHX15; enzalutamide

1| INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in American males. In 2018, it was estimated there would be 164 690 new prostate cancer cases and 29 430 deaths due to prostate cancer.[1] Androgen receptor (AR) plays a central role in normal prostate development as well as the development and progression of prostate cancer,[2–4] and androgen deprivation therapy (ADT) remains the standard treatment for patients with metastatic prostate cancer.[5] Unfortunately, despite an initial significant clinical response to ADT, most patients relapse with lethal castration-resistant prostate cancer (CRPC) after a mean time of 2–3 years.[6] AR activation under androgen depleted condition is the major mechanism driving CRPC development.[7,8] Therefore, highly potent second generation AR signaling targeting agents including abiraterone acetate and enzalutamide have been developed to inhibit AR signaling in CRPC.[9–11] However, both initial and acquired resistance to the second-generation AR targeting agents are common, which leads to prostate cancer mortality. Understanding mechanisms of this resistance is of great clinical significance.

According to studies by multiple investigators, the resistance mechanisms can be stratified into “AR-dependent” and “AR-independent” mechanisms.[12–14] AR-independent resistance mechanisms include glucocorticoid receptor overexpression, neuroendocrine differentiation, and immune system deregulation.[15–18] AR-dependent resistant mechanisms are the major mechanisms and include AR amplification, hyperactivation, mutation, expression of constitutively active AR splice variants, abnormalities in AR co-factors, DNA methylation of promoter, and intratumoral androgen biosynthesis.[12,19–23] Novel approaches to inhibit AR signaling in prostate cancer cells may result in new therapeutics for CRPC.

Through a yeast mutagenesis screen, we found PRP43 as a potential factor regulating AR subcellular localization and function.[24] The mammalian ortholog of Prp43 is DHX15, a member of the DEAH-box (DHX)RNA helicase family that can regulate a lot of biological processes, including RNA metabolism, cellular differentiation and apoptosis.[25]

Dysregulation of RNA helicase family proteins is commonly observed in tumor initiation and development.[26,27] However, specific functions of individual members of RNA helicase family remain elusive. DHX15 was reported to act as a tumor suppressor in glioma,[28] participate in immune response,[29] regulate intestinal antiviral innate immunity through activation of the Nlrp6-interferon pathway,[30] and inhibit the migration and proliferation of gastric cancer.[31] These findings suggest that DHX15 can have diverse functions in different types of cells. We recently reported that DHX15 can act as a novel AR coactivator in prostate cancer cells.[24] In our study, DHX15 could stabilize Siah2 levels and enhance its E3 ubiquitin ligase activity, subsequently resulting in the activation of AR. In hormone naïve prostate cancer specimens, DHX15 was upregulated and its expression was correlated with Gleason score and prostate-specific antigen (PSA) recurrence, suggesting that DHX15 could potentially contribute to the progression prostate cancer.[24] However, the role of DHX15 in prostate cancer, particularly in CRPC, remains to be fully elucidated. In the present study, we evaluated DHX15 expression in CRPC specimens and explored DHX15 regulation of AR signaling in the CRPC cell line C4–2, potential DHX15 modulation of enzalutamide resistance, and physical interaction between DHX15 and AR.

2| MATERIALS AND METHODS

2.1| Plasmid constructs

Full-length or part of DHX15 coding region was amplified by PCR using Myc-DHX15 as template and then cloned into the SacI and KpnI sites of pEGFP-C1 to construct GFP tagged GFP-DHX15, GFP-DHX15 [amino acids (aa) 1–475] and GFP-DHX15 (aa 476–795). Then GFPDHX15 (aa 1–322) and GFP-DHX15 (aa 323–475) were generated from GFP-DHX15 (aa 1–475) by deletion mutagenesis using Q5® Site-Directed Mutagenesis Kit (E0554S, New England Biolabs) according to the manufacture's protocol. Similarly, GFP-DHX15 (aa 476–661) and GFP-DHX15 (aa 629–795) were generated from GFP-DHX15 (aa 476795). All constructed plasmids were sequence confirmed.

2.2| Cell culture

For cell culture, cell lines 22Rv1, PC3, DU145, HEK 293 (ATCC), and C4–2 (kind gift from Leland K. Chung) were cultured in RPMI-1640 (10040-CV, Corning Cellgro) or Dulbecco's modified Eagle's medium (12–604F, Lonza) supplemented with 10% fetal bovine serum (FBS) (S11150, Atlanta Biologicals), 5% antibiotics and 1% L-glutamine at 37°C with 5% CO_2. Cells were cultured in phenol red-free medium (17105-CV, Corning Cellgro) supplied with 10% dextran coated charcoal-stripped FBS before treatment with dihydrotestosterone (DHT, Sigma-Aldrich). Dextran coated charcoal-stripped FBS was prepared by incubating 0.25% Norit-A charcoal (Sigma-Aldrich) overnight in 0.0025% Dextran T-70 (Sigma-Aldrich) in 0.25 M sucrose, 1.5 mM MgCl2, 10 mM HEPES, pH 7.4 at 4°C, followed by incubation in FBS (253 mg charcoal/100 mL FBS) for 12 h at 4°C. Cells were verified as mycoplasma free by PCR.[32] Cell lines 22Rv1, DU145 and C4–2 were authenticated in 2016 and 2017 using DNA fingerprinting by examining microsatellite loci in a multiplex PCR reaction (AmpFlSTR® Identifiler® PCR Amplification Kit, Applied Biosystems, Foster City, CA) by the University of Pittsburgh Cell Culture and Cytogenetics Facility. HEK 293

and PC3 cell lines were obtained from ATCC in 2016. ATCC performed authentication for HEK 293 and PC3 cell lines using short tandem repeat profiling.

For overexpression experiments, HEK 293 cells were transiently transfected with indicated expression vector(s) using PolyJet In Vitro Transfection reagent (SL100688, SignaGen Laboratories) according to the manufacturer's instructions. Cells were harvested and prepared for subsequent experiments 48 h after transfection.

Enzalutamide-resistant 22Rv1 cells (Enz^R-22Rv1) were generated by culturing 22Rv1 cells in complete RPMI-1640 media in the presence of 10 μM enzalutamide for several months (as described previously[33]). Once cells became resistant, they were maintained in complete RPMI1640 media containing 25 μM enzalutamide.

For knockdown experiments, cells were transfected with control siRNA (sc-37007 Santa Cruz) or siRNAs targeting DHX15 using DharmaFECT siRNA transfection reagent (T-2001–03, Dharmacon). The final concentration of siRNA was 50 nM in each well. After transfection for 48 h, the cells were suspended by trypsin (25–053-CI, Corning) and counted for colony formation assay or treated with different concentration of DHT as indicated for another 48 h. The DHT treated cells were directly lysed and analyzed by Western blot.

The siRNA targeting DHX15 were as follows: siDHX15.1, 5′-UCUGGAUAACUGUUCGAAUUGCUGCUU-3′; siDHX15.2, 5′-AUCUGUAAACCUAUCCUUGUAUUCCCA-3′ as previously.[24]

2.3| Western blot analysis

Cells were lysed in modified radioimmune precipitation assay (RIPA) buffer [50 mM Tris-Cl (pH7.4), 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl] with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined by BCA assay (Pierce Chemical Co., Rockford, IL). Western blotting was conducted using primary antibodies against AR (sc-816), PSA (sc-7638), DHX15 (sc-271686) and GAPDH (sc-47724) from Santa Cruz Biotechnology, and GFP (TP401, Chemokine and MA1952, Thermofisher Scientific) followed with horseradish peroxidase-labeled secondary antibody (172–1011, 170–6515, Bio-Rad, and sc2354, Santa Cruz Biotechnology). Signals were visualized using chemiluminescence (ECL Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and detected by ChemiDoc™ Imaging Systems (Bio-Rad, Hercules, CA).

2.4| Colony formation assay

Cells (1×10^3 or 2×10^3) were seeded into 6-well plates with medium containing 0–100 nM DHT or 0–10 μM MDV3100 as indicated. The colonies formed 8–15 days later were stained with 0.5% crystal violet, and colonies with a diameter equal to or larger than 0.5 mm were counted by ImageJ software (NIH, USA). Each cell growth and colony formation assay was carried out in triplicate and repeated three times.

2.5| Co-immunoprecipitation

For co-immunoprecipitation, HEK 293 cells in 10-cm culture plates were transiently transfected with 6 μg of the indicated plasmids, cultured for 48 h after transfection, and lysed in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 1.5 mM MgCl2, 1% NP-40, 15% glycerol, 2 mM EDTA) with protease inhibitor cocktails (P8345–5ML, Sigma) at a dilution of 1:100. After precleaning cell lysates with protein A/G plus-agarose beads (sc-2003, Santa Cruz) for 1 h and blocking anti-GFP (D153–8, MBL)/anti-Myc antibody-conjugated agarose beads (16–219, Millipore Sigma) with 2.5% albumin/bovine (94349–60-7, Acros organics) for 1 h, cell lysates were added to anti-Myc/anti-GFP antibody-conjugated agarose beads and rotated at 4°C overnight. The beads were washed using lysis buffer four times. Immunoprecipitates and total cell lysates were boiled in SDS loading buffer for 10 min and then subjected to Western blot analysis using anti-GFP antibody (TP401, Chemokine and MA1–952, Thermofisher Scientific) and anti-AR antibody (sc-816, Santa Cruz).

2.6| Immunohistochemistry and tissue microarray analysis (TMA)

The TMAs containing CRPC specimens were obtained from Prostate Cancer Biorepository Network (PCBN) and Duke University (Dr. Jiaoti Huang). Use of these prostate tissues was approved by the PCBN and the University of Pittsburgh Institutional Review Board. Immunohistochemical staining was performed on the paraffin embedded TMA using DHX15 antibodies (sc-271686, (E-6), Santa Cruz Biotechnologies) as described previously.[34] DHX15 expression was assessed as a function of staining intensity and percentage of cells exhibiting each level of intensity, as previously.[34] The intensity of the reaction product was based on a 4-point scale: none, faint/equivocal, moderate, and intense. An H-score was calculated using the following formula: H-score = 0(% no stain) + 1(% faint/equivocal) + 2(% moderate) + 3(% intense).TMAs were scored by three investigators in a blinded fashion (YDX, FMD, LEP).

2.7| Statistical analysis

All graphs were generated using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). The data are presented as mean ± SEM. Statistical analyses were performed with Student's *t*-test. $P < 0.05$ was considered statistically significant.

3| RESULTS

3.1| DHX15 expression was upregulated in CRPC specimens

To evaluate the potential role of DHX15 in CRPC, we performed DHX15 immunostaining of two tissue microarrays (TMAs) of prostate cancer specimens containing CRPC, one set from the Prostate Cancer Biorepository Network (PCBN) and another from Duke University. We were able to generate DHX15 staining in 7 of the 21 hormone naïve prostate cancer and 20 of the 38 CRPC cores in the TMAs. DHX15 showed a nuclear expression pattern in both hormone naïve and CRPC specimens (Figure 1A). DHX15 expression was upregulated in CRPC samples compared to hormone naïve tumor samples ($P = 0.0126$) (Figure 1B).

3.2| DHX15 knockdown reduced AR sensitivity to low DHT concentrations in C4–2 cells

As an AR co-factor, DHX15 may sensitize the responsiveness of AR to androgens. The C4–2 cell line was reported to exhibit hypersensitivity to DHT,[35,36] providing an excellent model to test the effect of DHX15 knockdown on AR activity at low DHT concentrations. Following siDHX15 knockdown, C4–2 cells were cultured in charcoal-stripped medium containing DHT at concentrations ranging from 0 to 100 nM and the expression pattern of three AR target genes, ELL2, EAF2, and PSA, was determined. We did not treat cells with DHT at concentrations higher than 100 nM, because 100 nM is already much higher than physiological level of DHT (0.38–3.27 nM).[37] In the presence of control siRNA, the maximal expression of androgen-response genes was induced at 1 nM DHT for EAF2 or 10 nM DHT for ELL2 and PSA (Figure 2A). In the presence of siRNA targeting DHX15, the maximal induction of androgen-response genes was observed when the cells were cultured in the presence of 100 nM DHT (Figure 2A). This observation suggested that DHX15 knockdown caused a shift of the DHT concentration required for stimulating maximal expression of androgen-response gene expression from 1 to 10 nM to 100 nM or greater. To exclude the off-target effect of siRNA targeting DHX15, we also inhibited DHX15 expression using another siRNA targeting DHX15 and observed a similar result (Figure 2B).

We also measured the colony formation for C4–2 cells in response to various DHT concentrations with or without DHX15 knockdown (Figure 3A). Quantification of C4–2 colonies resulted in a bell-shaped growth in response to increasing DHT concentrations (Figure 3B). This result was consistent with the bell-shaped growth curve in response to increasing DHT concentrations in the LNCaP model.[38–40] The bell-shaped growth curves were observed in the presence of either control siRNA or siRNA targeting DHX15 (Figure 3B). However, DHX15 knockdown inhibited C4–2 cell growth when DHT concentrations were at 0.1 nM or lower but not at 1 nM or higher DHT concentrations. This result suggests that DHX15 is important for C4–2 cells growth in the presence of castrate levels of androgens.

3.3| DHX15 knockdown enhanced the sensitivity of C4–2 cells to enzalutamide (MDV3100)

AR co-regulators have been postulated as potential drivers of enzalutamide resistance in prostate cancer cells.[41] Since DHX15 was identified as an AR coactivator,[24] it may play a role in enzalutamide resistance in prostate cancer cells. Thus, we tested if DHX15 knockdown could enhance the sensitivity of prostate cancer cells to enzalutamide using C4–2 as a model, since C4–2 is a CRPC cell line derived from LNCaP.[42,43] After DHX15 knockdown, C4–2 cells were subjected to colony formation assay in the presence of enzalutamide (0–10 μM) (Figure 4A, Supplemental Figure S1A). The cells were cultured in RPMI-1640 complete medium supplemented with 10% dextran-coated charcoal stripped FBS, which contains a castrate level of testosterone. As expected, the colony number of C4–2 cells decreased in the presence of enzalutamide in a dose dependent manner for both DHX15 knockdown (siDHX15) and control (siNC) groups (Figure 4A). For the control group, the normalized colony formation in the 0.1, 1, and 10 μM enzalutamide treatment groups were 0.88, 0.78, and 0.55, respectively. In comparison, the normalized colony formation in the presence of 0.1, 1, and 10 μM enzalutamide was 0.70, 0.49, and 0.33 for the DHX15 knockdown groups, respectively (Figure 4B). DHX15 knockdown enhanced

enzalutamide inhibition of C4–2 colony formation by 20–30%. However, we did not observe the same phenomenon in another AR-positive cell line, 22Rv1, which was less sensitive to enzalutamide possibly due to its expression of constitutively active AR splice variants (Figure 4C, Supplemental Figure S1B). The sensitivity of 22Rv1 to enzalutamide in culture was not affected by DHX15 knockdown (Figure 4D). We also tested the effect of DHX15 knockdown on enzalutamide sensitivity in enzalutamide-resistant 22Rv1 (Enz^R-22Rv1) cells. The sensitivity of Enz^R-22Rv1 to enzalutamide in culture was not affected by DHX15 knockdown (Supplemental Figure S2). We also performed the same assay in two AR-negative prostate cancer cell lines, PC3 and DU145. As expected, enzalutamide had no significant effect on proliferation of these two cell lines in the presence siNC or siDHX15 (Figure 4E and H, Supplemental Figure S1C, D).

3.4| AR co-immunoprecipitates with the N-terminal region of DHX15

Our previous results showed that DHX15 co-immunoprecipitated with AR and can regulate AR transcriptional activity.[24] Four conserved domains in DHX15 were predicted by NCBI's Conserved Domain Database (CDD), which are DEXDc superfamily (149–322), HELICc (382–475), HA2 (545–628), and OB_NTD_bind (662–765). In this study, we constructed four DHX15 deletion mutants based on the predicted domains to explore which segment of DHX15 was necessary for the interaction with AR. GFP-DHX15 (full length), GFP-DHX15 (aa 1–322), and GFP-DHX15 (aa 323–475) co-precipitated with Myc-AR using anti-Myc antibody-conjugated agarose beads. However, GFP-DHX15 (aa 476–661) or GFP-DHX15 (aa 629–795) were barely co-precipitated with Myc-AR in the same experiment (Figure 5A). Similar results were obtained when the same transfected cell lysates were co-precipitated using anti-GFP antibody-conjugated agarose beads. Myc-AR was coprecipitated efficiently with GFP-DHX15 (full length), GFP-DHX15 (aa 1–322), and GFP-DHX15 (aa 323–475), but barely with GFP-DHX15 (aa 476–661) or GFP-DHX15 (aa 629–795) (Figure 5B). These results suggested that the N-terminal domain of DHX15 (aa 1–475) plays a major role in DHX15 interaction with AR.

4| DISCUSSION

Our results suggest that DHX15 may play an important role in prostate cancer progression to castration resistance. DHX15 immunostaining appeared to be up-regulated in human CRPC specimens compared to hormone naïve specimens. In a CRPC prostate cancer cell line model, DHX15 knockdown reduced AR sensitivity to DHT and inhibited cell growth only when DHT levels were at 0.1 nM or lower. In addition, DHX15 knockdown enhanced enzalutamide inhibition of C4–2 cell growth. These observations together suggest that DHX15 upregulation could contribute to prostate cancer progression to castration resistance.

DHX15 appears to be more important for CRPC cell growth when DHT concentrations are low. Our studies showed that DHX15 knockdown reduced C4–2 cell growth when DHT levels were at 0.1 nM or lower but not when DHT level was at 1 nM or higher. Low DHT levels can be stimulatory to prostate cancer cell growth whereas supraphysiological DHT levels are often growth suppressive.[38,40] Since DHX15 appears to be an AR co-activator, DHX15 knockdown should reduce the efficacy of androgens to stimulate AR. As expected,

when DHT levels were ≤0.1 nM, DHX15 knockdown reduced the growth stimulatory effect of DHT in C4–2 cells. Similarly, when DHT levels were 1 nM or above, DHX15 knockdown reduced the growth inhibitory effect of DHT in C4–2 cells. Thus, the elevated DHX15 expression in CRPC specimens could sensitize AR to castrate levels of androgens and stimulate CRPC growth.

Supraphysiological androgen suppression of CRPC was observed and represents a potential treatment for CRPC patients.[44–48] However, it is not clear why supraphysiological androgen therapy is only effective in some CRPC patients. The AR transcriptional activity and growth showed bell-shape curves in response to increasing DHT concentrations in C4–2 provides a potential model for studying supraphysiological suppression of CRPC. The expression levels of AR target genes increased along with the increasing level of DHT at first, and then decreased at supraphysiological DHT concentrations. However, the suppression of androgen-response gene expression by high levels of DHT was not observed when DHX15 was knocked down. Also, the growth suppression of C4–2 by high DHT was partially alleviated by DHX15 knockdown. This suggests that CRPC with low DHX15 may be less sensitive to supraphysiological DHT suppression. Future studies will be needed to address the potential role of DHX15 in supraphysiological androgen suppression of prostate cancer.

The effect of DHX15 knockdown to sensitize cells to enzalutamide appears to be mediated through AR, particularly the LBD domain that binds to DHX15.[24] Although DHX15 knockdown could inhibit the growth of AR-negative DU145 and PC3 cells in colony formation assay, the sensitivity of these AR-negative cells to enzalutamide was not affected by DHX15 knockdown. Also, DHX15 knockdown did not influence the effect of enzalutamide on both parental and enzalutamide-resistant 22Rv1 cells, which express both full-length AR and AR splice variants lacking LBD. The AR splice variants are thought to drive enzalutamide resistance in 22Rv1 cells because these AR variants do not respond to enzalutamide that targets LBD. In our previous studies, we reported that DHX15 interact with LBD of AR.[24] Therefore, DHX15 knockdown should not affect AR splice variants and enzalutamide sensitivity in 22Rv1 cells. This observation argues that DHX15 knockdown modulation of prostate cancer cells response to enzalutamide is mediated through AR, particularly its LBD.

Using colony formation assay, here we showed that DHX15 knockdown could also inhibit cell growth in AR-negative cells (Supplemental Figure S3). This finding is not surprising, considering that DHX15 is a component of splicesome and involved in RNA splicing. Ito S, et al. reported that DHX15 acted as a tumor suppressor in glioma[28]; Xiao YF, et al., discovered its role in the inhibition of migration and proliferation role in gastric cancer.[31] These findings suggest that DHX15 can modulate multiple signaling pathways in addition to AR. However, we previously did not detect inhibition of DU145 and PC3 cells proliferation by DHX15 knockdown using a BrdU assay.[24] One potential reason for the difference is that colony formation assay can detect long-term and cumulative effects of DHX15 knockdown on cell growth and cell death, whereas BrdU assay only detects short-term effects of DHX15 knockdown on DNA synthesis. We compared BrdU and colony formation assay in DU145 and PC3 cells. BrdU assay did not detect the effect of DHX15 knockdown on DU145 (data not shown), which was detected by colony formation assay. Thus, we decided to use colony

formation assay in this study. Another possibility that could affect reproducibility is phenotype drifting of cultured cells and we cannot rule out this possibility since the PC3 cells used in our previous studies were not authenticated. To minimize this potential variable, all the cells used in this study were recently purchased or authenticated.

5| CONCLUSIONS

In summary, these studies suggested that DHX15 is likely to play an important role in prostate cancer progression to castration resistance via enhancing AR activity. Targeting DHX15 should inhibit AR activity and enhance the efficacy of antiandrogens in CRPC. However, inhibition of DHX15 may have side effects because DHX15 is also involved in other pathways important for cell growth. One potential approach will be to develop small molecules that can specifically disrupt interactions between AR and DHX15. This study showed that AR interacts with the N-terminus of DHX15. Future studies to define interactions between AR and DHX15 may lead to novel approaches to specifically target DHX15-AR interaction in CRPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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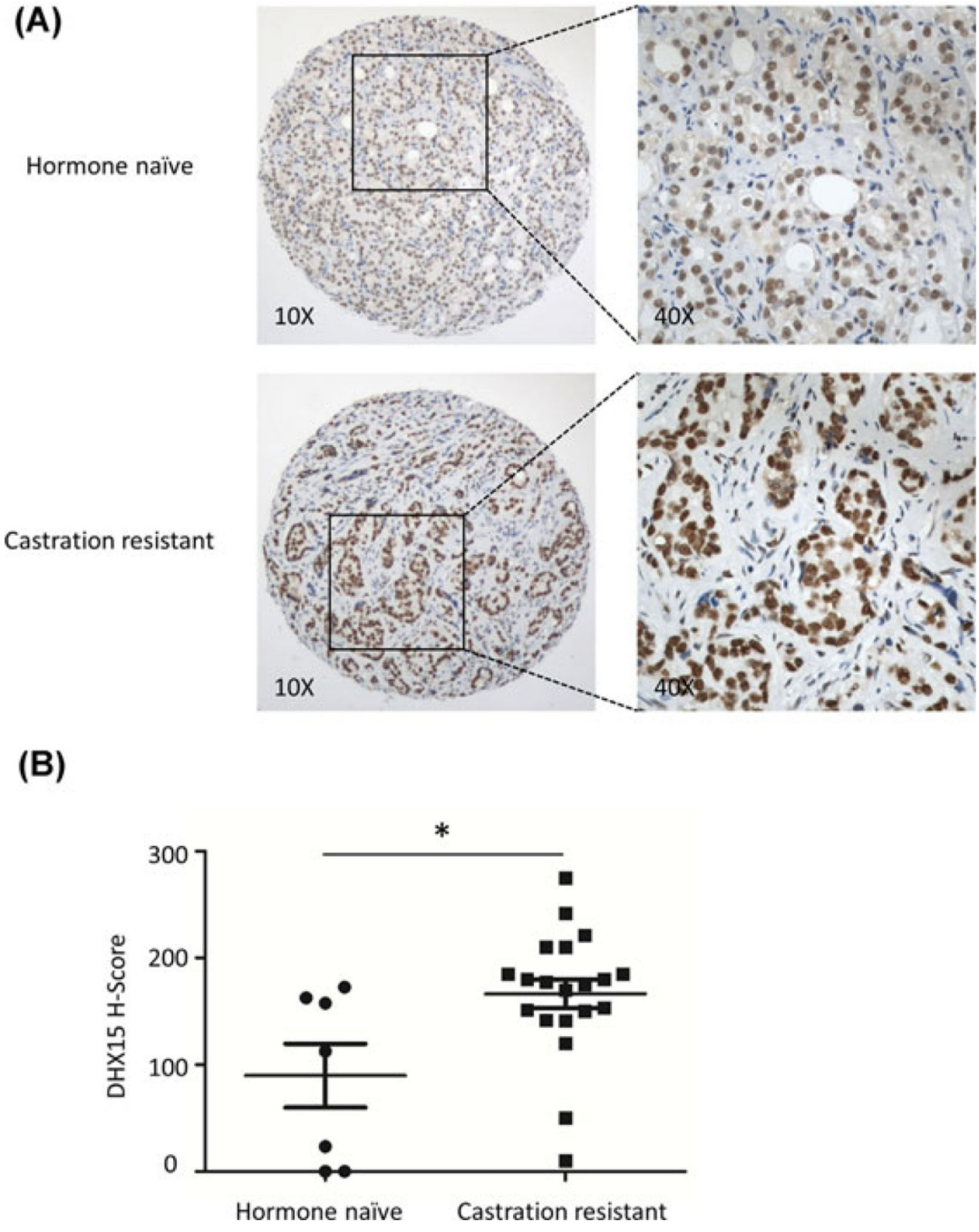

FIGURE 1.

DHX15 is upregulated in CRPC specimens compared to hormone naïve specimens. (A) Representative images of DHX15 IHC staining from the PCa TMAs. (B) Quantification of DHX15 IHC staining on the Hormone naïve and CRPC samples. $P = 0.0126$.

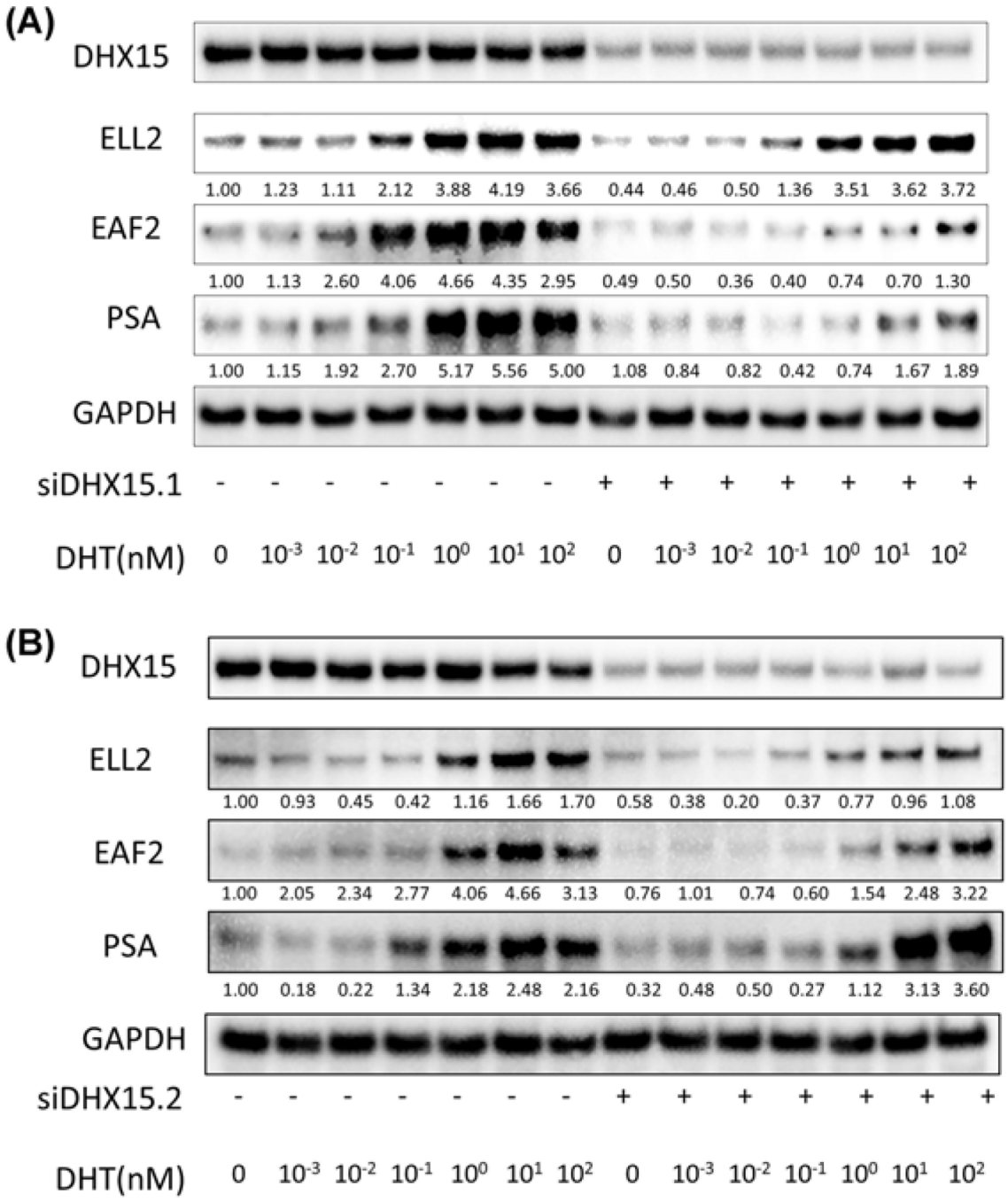

FIGURE 2.

DHX15 knockdown shifted the androgen dose response towards higher DHT concentrations in C4–2 cells. (A) C4–2 cells were transfected with Non-target Control siRNA (siNC) or siDHX15.1 targeting DHX15 for 48 h in charcoal-stripped medium. Then the cells were treated without or with the indicated concentration of DHT for another 48 h. Then the cell lysate were immunoblotted with anti-DHX15, ELL2, EAF2, PSA, and GAPDH antibody. (B) Another siRNA targeting DHX15 (siDHX15.2) was used to avoid the off-target effect. The experiment was conducted as described in (A)

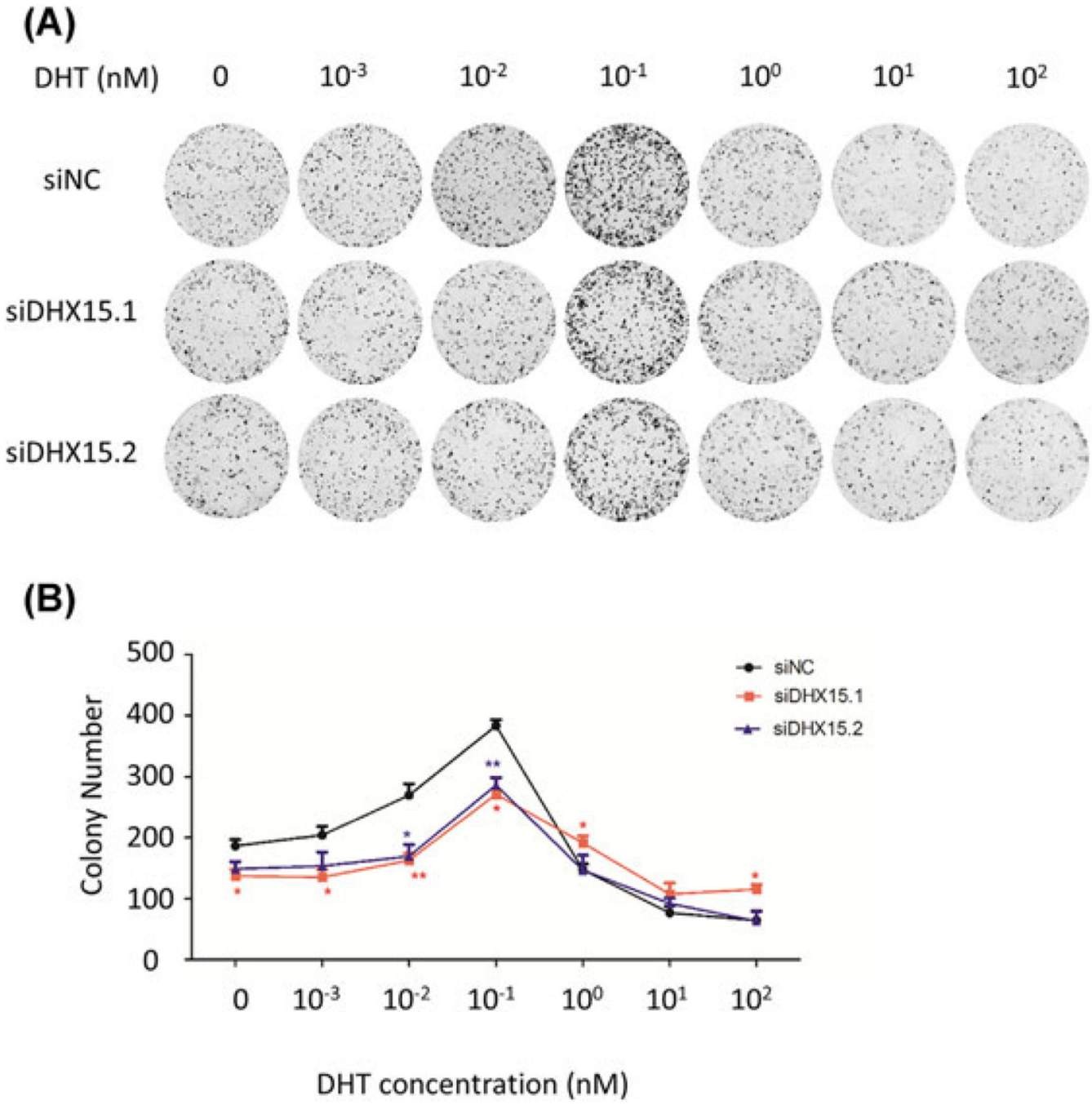

FIGURE 3.

Knockdown of DHX15 attenuated C4–2 cells responsiveness to DHT. (A) Non-target control siRNA (siNC) or siRNAs targeting DHX15 (siDHX15.1 and SiDHX5.2) transfected C4–2 cells were seeded at 2000 cells per well in 6-well plates in triplicate. After 12 days cultured in charcoal-stripped medium without or with the indicated concentration of DHT, cell colonies were fixed by 10% formalin and then stained with 0.1% crystal violet for 20 min. The colony images are representative of three independent experiments. (B) The colony

number was quantified by ImageJ software. Data was shown as mean ± SEM. (*$P < .05$, **$P < .01$).

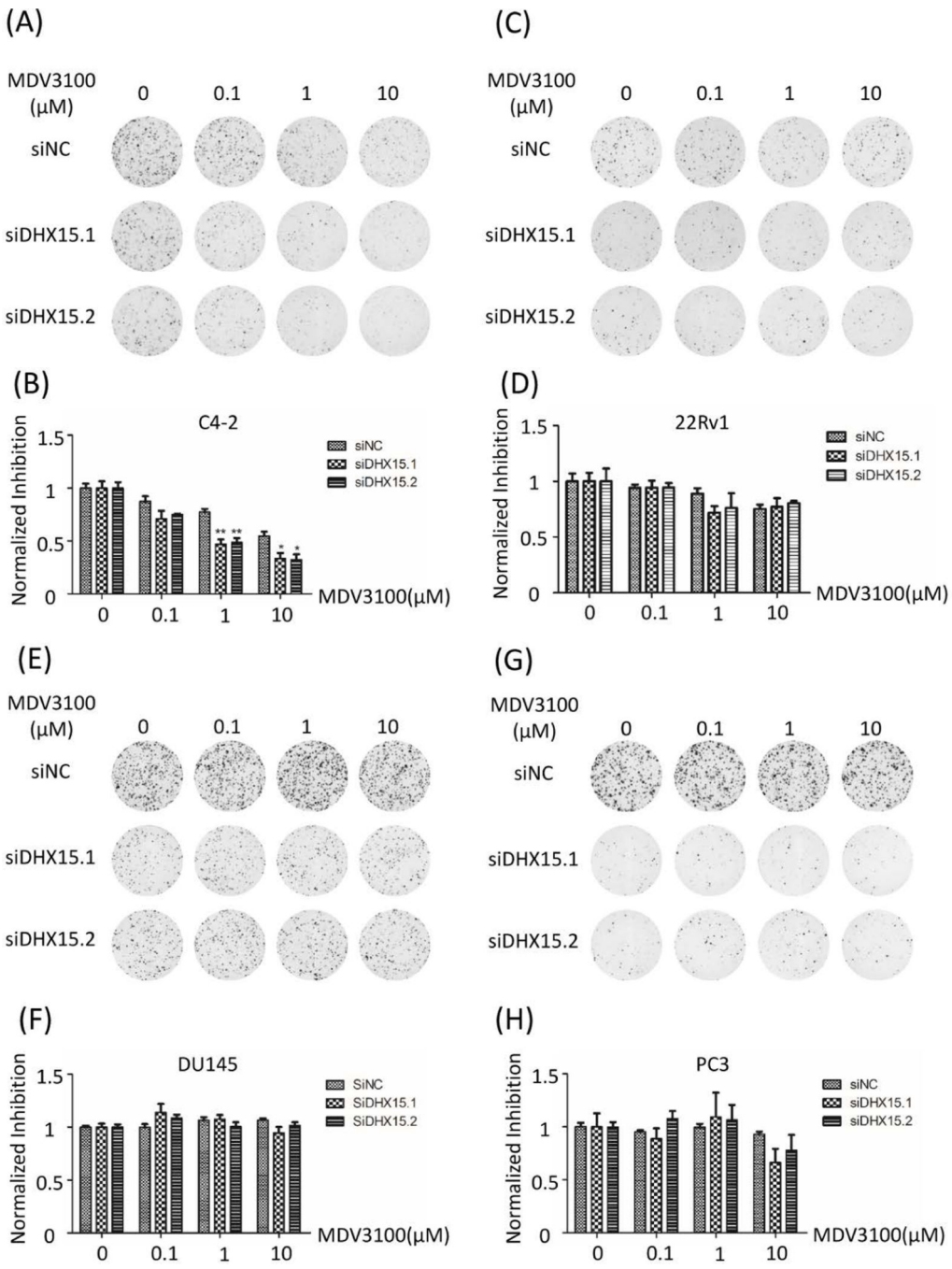

FIGURE 4.

DHX15 knockdown enhances the sensitivity of C4–2 cells to enzalutamide (MDV3100). (A) C4–2 cells were transfected with non-target control siRNA (siNC) or two different siRNAs targeting DHX15 (siDHX15.1 and siDHX15.2) for 48 h. Then cells were seeded to in 6-well plates at 1000 cells per well in triplicate and treated with four different concentration of MDV3100 as indicated. After 12 days culture, cell colonies were fixed by 10% formalin and then stained with 0.1% crystal violet for 20 min. The colony images are representative of three independent experiments. (B) The normalized inhibition represents the percentage of

cells left in each treatment group compared with vehicle group. Data was shown as mean ± SEM. (C), (E), (G). 22Rv1(C), DU145(E), PC3(G) cells were transfected with siNC, siDHX15.1 and siDHX15.2 for 48 h. Then the cells were subjected to colony formation assay as described in (A). 22Rv1 was cultured for 15 days, DU145 was cultured for 8 days and PC3 was cultured for 12 days. (D), (F), (H). The normalized inhibition for 22Rv1(D), DU145(F) and PC3(H) cells treated with enzalutamide respectively. (*$P < .05$, **$P < .01$).

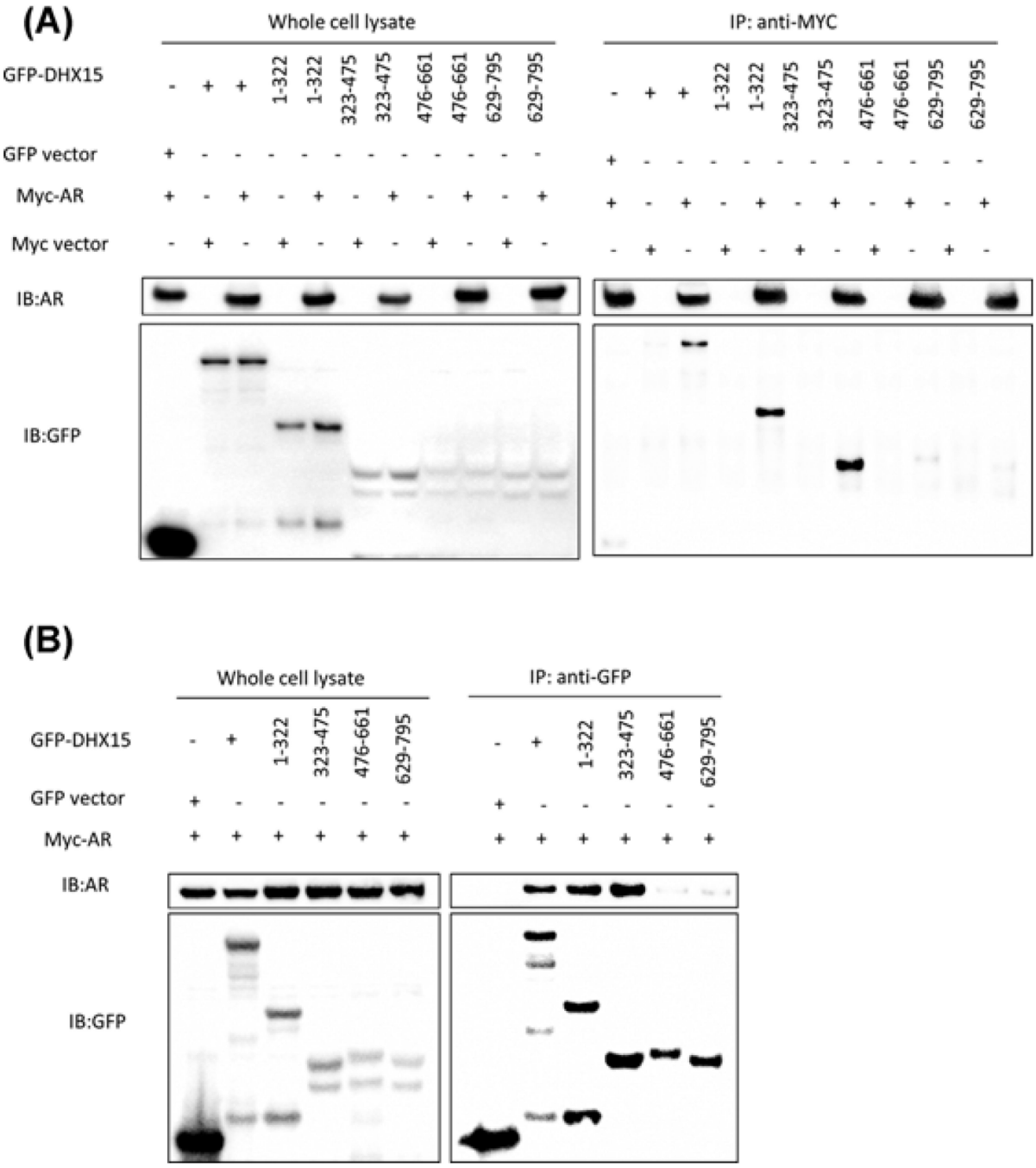

FIGURE 5.

Co-immunoprecipitation of DHX15 with AR. (A) HEK 293 cells were transfected with Myc-tagged wild type AR and GFP-tagged wild type DHX15 and DHX15 deletion mutants (aa 1–322, 323–475, 476–661, 629–795) as indicated for 48 h. Cell lysates were immunoprecipitated with antibodies against Myc. The associated proteins were immunoblotted with GFP antibody. (B) HEK 293 cells were transfected with Myc tagged wild type AR and GFP tagged wild type DHX15 and DHX15 deletion mutants (aa 1–322, 323–475, 476–661, 629–795) as indicated for 48h. Cell lysates were immunoprecipitated

with antibodies against GFP. The associated proteins were immunoblotted with AR antibody. Data are representative of three different experiments